THE INVOLVEMENT OF ESTROGEN RECEPTORS IN ASTROCYTE SURVIVAL

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COLUMBUS STATE UNIVERSITY

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A THESIS SUBMITTED TO THE

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ABSTRACT

Estrogens are a class of hormones that are demonstrated to be neuroprotective. The levels of estrogen in the body decline during menopause which can cause a variety of symptoms. Hormone replacement therapy (HRT) is a treatment option for menopause, however, it has been demonstrated to have adverse side effects. Of the treatment options for adverse side effects of HRT, Selective estrogen Down-regulators do not appear to have an agonist effects on estrogen receptors while Selective estrogen receptor Modulators do. The aim of this study was to determine the role that estrogen receptors play in the survival of astrocytes when placed under stress with epinephrine as well as when treated with a well-known SERD. The results of this experiment indicated that treatment with estrogens did not offer any neuroprotection. Future studies should focus on differing concentrations of variables and different exposure times to further evaluate the mechanisms of estrogen signaling.

INDEX WORDS: estrogen, estrogen receptors, menopause, hormone replacement therapy

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Background

Menopause is a natural life transition for women that are between 40 and 50 years of age. Women normally experience symptoms including, but not limited to, hot flashes, mood changes, and decreased sex drive. These symptoms are due to a decrease in the production of the female hormones estrogen and progesterone (De Souza and Ogava, 2014). Estrogens are a class of hormones that are found naturally in both sexes and have various physiological effects. Aside from their reproductive function, estrogens help regulate the metabolism of lipids and carbohydrates as well as influence the cardiovascular and neurological systems (Vrtacnik et. al., 2014). The treatment that is often used to help alleviate the symptoms of menopause is hormone replacement therapy (HRT). However, studies have been conducted that demonstrate that HRT can have adverse side effects such as increased risk of stroke, breast cancer and cardiovascular disease (Schmidt et al., 2006). The preferred treatment option is oral conjugated equine estrogens (CEE) as they are cost effective and convenient. However, despite this benefit, they have the above mentioned side effects (Schmidt, 2006). Due to the adverse side effects of CEE, it is recommended to minimize estrogen replacement by administering the lowest effective dose with the shortest possible duration. The central nervous system acclimates to the decrease in estrogen levels over time in many postmenopausal women, causing a natural decline of menopause symptoms (Schmidt et al., 2006).

The most dominant and potent estrogen is 17 beta-estradiol, although there are small amounts of 17 alpha-estradiol, estrone and estriol present in the body as well (Björnström and Sjöberg, 2005). The reproductive functions of estrogens are to promote the expression of primary and secondary sex characteristics in females. They also have effects on male physiology as well as cardiovascular and central nervous system health (Vrtacnik et. al, 2014). Due to their role in

the central nervous system, the decline in estrogen levels during menopause can cause women to experience symptoms of memory loss, depression, and possibly more serious disorders such as Alzheimer's disease (De Marinis et al., 2011). Studies have shown that estrogens have a protective action on the brain and, with early intervention, estrogen therapy can help decrease the vulnerability to neurodegenerative diseases (Wang et al., 2006). Grimes and Hughes (2015) conducted an *in vitro* study to determine the neuroprotective effects of 17-beta estradiol, and two CEEs; equilin and equilenin at varying concentrations against oxidative stress on astrocyte cells. Their results indicated that CEEs offer some degree of protection against oxidative stress for short term exposure. However, when exposed to a longer period of oxidative stress, CEEs and 17-beta estradiol were not as effective in preventing cell death (Grimes and Hughes, 2015).

Hormone replacement therapy has been implemented as a treatment option for the declining levels of estrogens during menopause. This treatment option has been shown to help alleviate several symptoms of menopause including hot flashes, vaginal dryness and a decreased risk of osteoporosis (Souza and Ogava, 2014). Oral conjugated equine estrogens (CEEs), such as Premarin (Pfizer), contain several different estrogens from the urine of pregnant mares including hormones that are not naturally producing in humans (Zhao and Brinton, 2006). The composition is made up of sulfate esters of classical estrogens and ring-beta unsaturated estrogens that include equilin and equilenin. While the goal of HRT is help increase estrogen levels in the body and has several benefits, studies have shown that CEEs can have negative side effects as well, which include an increased risk of ovarian, breast and endometrial cancer (Souza and Ogava, 2014).

There are notable differences between endogenous human estrogens, particularly estradiol, and the components of CEEs. Estradiol primarily regulates its effects through estrogen receptor (ER) alpha, while some of the estrogens found in CEEs are mediated by ER beta

(Bhavnani and Stanczyk, 2014). Estrogen receptors have different effects on the body and are expressed at different levels in specific tissues in the body. ER alpha is primarily found in the uterus, breast tissue, adipocytes and kidneys, while ER beta is prevalent in the colon, lungs and bladder. Both receptors are found at varying levels in testes, ovaries and the brain (Vrtacnik et. al, 2014).

Astrocytes are the predominant form of glial cells in the brain and spinal cord. They are supportive cells for neurons and are involved in the formation of the blood brain barrier as well as synaptic transmission between neurons. Another important function that they have in the central nervous system is regulation of hormones, particularly in female reproduction (Micevych, et al., 2010). Ovulation occurs in females when there is a spike in luteinizing hormone (LH), which is triggered by gonadotropin releasing hormone (GnRH) from the hypothalamus. LH is directly influenced by estradiol, which has a positive feedback on both LH and GnRH. Neurons involved in the release of GnRH do not have estrogen receptors; estradiol must first stimulate astrocytes to control the expression of GnRH (Micevych et al., 2010).

Along with regulation of ovulation, the astrocyte-estrogen relationship is involved in neuroprotection against dementia and other neurodegenerative diseases. Studies have shown that estrogen mediates glutamate uptake. Glutamate is a neurotransmitter involved in cognition but at high levels can be toxic to neurons, which is the reason that declining estrogen levels can lead to dementia (Liang et al. 2002). The concentration of glutamate in the cell is regulated by glutamate transporters that remove the neurotransmitter from the extracellular space. Estradiol has been shown to increase the amount of glutamate transporters on astrocytes, therefore providing neuroprotection and decreasing the vulnerability to neurodegenerative diseases (Lan, et al. 2014).

Estrogen influences neural physiology by targeting neuronal and glial cells found in the brain. Mechanisms of estrogen signaling include direct and indirect genomic signaling, non-genomic signaling and ligand-independent signaling. Estrogen signaling occurs through two different nuclear estrogen receptors, ER alpha and ER beta (Henderson, 2007). The classic model of estrogen signaling is direct genomic signaling, involving the nuclear receptors ER-alpha and ER-beta (Vrtacnik et. al, 2014). In direct genomic signaling, estrogens first bind to a ligand receptor in the nucleus, which causes these receptors to polymerize and then bind to estrogen response elements (EREs) that are found within the target genes. Target genes that are affected include insulin-like growth factor (IGF), collagenase and cyclin D1. Estrogens target genes in the central nervous system that are related to promoting neuronal growth and survival through transcription regulation (Björnström and Sjöberg, 2005). Estrogen signaling can also occur through G protein-coupled estrogen receptor 1 (GPER1) which is located on the cell's plasma membrane and controls rapid estrogen signaling (Vrtacnik et al., 2014). Estrogen binds to GPER1 and activates adenylate cyclase and protein kinases that have several different effects on the body. GPER1 is different both structurally and genetically from both nuclear estrogen receptors (Vrtacnik et al., 2014).

Several studies have been conducted on drugs available for the treatment of menopause symptoms in women in regards to their interaction with the ER receptors, specifically ER alpha. A significant portion of these studies focus on the treatment of breast cancer with two therapies (Yeh, et al 2013). Selective estrogen receptor modulators (SERMs) are a common drugs of choice that can be either ER alpha agonists or antagonists, depending on the target tissue, and affect gene expression in various ways (Hadji, 2012). Tamoxifen has been a particularly successful SERM in regards to the treatment of breast cancer due to its antagonistic effects on

breast tissue. However, a side effect of the drug is endometrial cancer which demonstrates its agonist effects within uterine tissue (Hadji, 2012). The second type are Selective estrogen receptor Down-regulators (SERDs) which do not appear to show any agonist properties and down regulate ER expression by degrading the ER alpha protein (Yeh, et al 2013). The most common SERD is fulvestrant, which is an analog of 17 beta-estradiol that acts as a competitive inhibitor for the binding of 17 beta-estradiol to its receptor and consequently has a higher affinity for the binding site compared to tamoxifen. Unlike tamoxifen; which just affects transcription through the ER receptors, fulvestrant fully destroys the ER protein, leading to complete suppression of signaling of 17 beta-estradiol via the estrogen receptor (Yeh, et al., 2013).

This study will aim to determine the genomic mechanism of estrogen signaling through ER alpha when cells are placed under stress with epinephrine and how survival is influenced by the addition of fulvestrant when treated with CEEs. I hypothesize that astrocytes that are placed under stress will show decreased survival when treated with fulvestrant and estrogen compared to those that do not receive the treatment due estrogen is not able exert any neuroprotective effects once the receptor is degraded. Also, I predict that astrocytes that are treated with equilenin will show decreased viability in comparison to those treated with 17 beta-estradiol.

Methods

Cells

The cells used in this study were human cultured astrocytes (1321N1, Sigma Aldrich). Astrocytes were prepared in media and stored at 37° C and 5% CO_2 in an incubator and the media was changed every 2 to 3 days. Media consisted of 10 mL of Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum, 2mM L-glutamine and 1% antibiotic and antimycotic in T-75 flasks. When cells became confluent, media was removed and a 10 mL solution of trypsin-EDTA

was added to the flask to remove the adherent cells from the surface. The flask was placed back in the incubator for 15 minutes. After the cells had dispersed, the contents of the flask were placed in tube along with 10 mL of media and centrifuged at 3000 rpm for 3 minutes. The supernatant was discarded and the cell pellet was re-suspended in 5 mL of media. 200 µL of cell-suspension with a concentration of 1 x 10^6 cells/mL was added to each respective well (Grimes and Hughes, 2015). To determine the concentration of cells, a hemocytometer was used to manually count cells. A solution made of 10 µL of prepared astrocytes with an equal amount of trypan blue solution was mixed in a well. The addition of the trypan blue solution showed presence of live and dead cells. Using a micropipette, 10 µL of the trypan blue –cell suspension mixture was pipetted onto the hemocytometer with a coverslip applied. Using an inverted microscope, both dead and live cells were counted (cells/mL). The number of cells counted was then used to calculate the amount of cell suspension and media needed to dilute to a final concentration of 1 x 10^6 cells/mL. The total amount of media that was to be used was obtained by multiplying the number of wells used by the volume of media in each well and then adding an extra 200 µL to ensure that there would be enough media available. (12 wells x 200 µL = 2400 µL + 200 = 2600 µL used). Once the volume of cell suspension was calculated, this was subtracted from the total amount of media used to give the final amount of media that would be added with the cell suspension. The solution of cell suspension and media was added to the respective wells at 200 µL/well and treatments began the next day. The formula for the calculation is shown here:

$$(Volume\ of\ cell\ suspension\ in\ \mu L) = \frac{\left(1\ x\ 10\text{^}6\frac{cells}{mL}\right)(Total\ media\ used\ in\ \mu L)}{(\#\ of\ cells\ counted)}$$

Treatment of Astrocytes

In order to test the hypotheses about the effects of estrogens on astrocytes, cells were treated with a fixed concentration of 1 μM equilenin or 17-beta estradiol (Grimes and Hughes, 2015). Cells were either oxidatively stressed using 1 μM epinephrine for 1.5 hours or kept unstressed. The concentration of epinephrine was obtained from preliminary work conducted by a graduate student at Columbus State University who examined astrocyte exposure to varying levels of epinephrine. There were also wells with no estrogen treatment to use as controls. 20 μL of Fulvestrant (1 μM) was added one hour prior to the treatment with estrogens (Jansen et al., 2002). 20 μL of DMSO was added to wells without any fulvestrant. The astrocytes were then treated with 10 μL of estrogens for one hour, followed by the addition of 20 μL of epinephrine (10 μM) for 1.5 hours. For wells without estrogens or epinephrine, the same concentration of PBS was added. The media was changed after being treated with epinephrine and viability of the astrocytes was measured 24 hours later. Four trials were completed with the same steps listed above. The 96-well plate is diagrammed in Table 1.

Table 1. 96-well plate diagram for treatments. Cells were first treated with 20 μL of fulvestrant, 20 μL of DMSO was added to cells without fulvestrant. After 1 hour with fulvestrant treatment, cells were treated with 10 μL of equilenin or 17β estradiol, 10 μL of PBS was added to wells without any estrogen treatment. After 1 hour with estrogen treatment, cells were treated with 20 μM of epinephrine for 1.5 hours. Media was changed after epinephrine treatment. Equilenin (Q) and 17β estradiol (E): 1 μM; Epinephrine (Epi): 10 μM; Fulvestrant (F): 1 μM; (+) and (-) indicates presence or absence of the variable.

	1	2	3	4	5	6	7	8	9	10	11	12
	+Q		+E		+Q		+E		(No estrogen)		(No estrogen)	
A +F	+F +Q -Epi		+F +E -Epi		+F +Q +Epi		+F +E +Epi		+F -Q or E -Epi		+F -Q or E +Epi	
B												
C -F	-F +Q -Epi		-F +E -Epi		-F +Q +Epi		-F +E +Epi		-F -Q or E -Epi		-F -Q or E +Epi	

MTT Cell Proliferation Assay

After treatments were complete, viability was accessed using MTT Cell Proliferation Assay (Sigma Aldrich). The media was removed from each well and 100 µl was added to each respective well as well as 2 wells to use as blanks for comparisons. 10 µl of the MTT reagent was then added, followed by incubation of the astrocytes for 2 hours and then 100 µl of the MTT solubilization solution was added to the wells. Absorbance was recorded at 570nm, with a high absorbance value indicating greater cell viability.

Analysis of data

The independent variables that were measured were treatment of estrogens and fulvestrant and if astrocytes are stressed or not stressed with epinephrine. To evaluate statistical significance between treatments, a Tukey's post hoc test and 3-way Analysis of Variance (ANOVA) was conducted with a p-value < 0.05 indicating statistical significance. Four trials were completed with the same steps listed previously.

Results

The absorbance values obtained from the MTT Assay were averaged and then converted to a ratio with the control. There was no significant difference found for astrocytes that were treated with estrogens (17-beta estradiol and equilenin) and those that were treated without estrogens (0.89 +/- 0.63 for 17-beta estradiol and 1.2 +/- 0.72 for equilenin, p=0.540) and the two estrogens did not differ significantly from each other (p= .559) or the treatment without estrogen (p=0.651 for equilenin, p=0.988 for 17-beta estradiol). Figure 1 shows the average cell viability (+/- S.D.) for cells that were treated with estrogen alone, compared to the control which had no treatment. Cells that were treated with fulvestrant or epinephrine in addition to estrogen treatment showed no significant difference between those that were treated without fulvestrant or epinephrine

treatment and estrogen (p=0.144 for fulvestrant, p=0.634 for epinephrine). Figures 2 and 3 show a comparison of treatment with estrogen vs. cells treated with estrogen and epinephrine (1.4 +/- 1.1 for equilenin and 1.8 +/- 1.2 for estradiol, Figure 2) and cells treated with estrogen and fulvestrant (1.3 +/- 1.4 for equilenin and 0.67 +/- 0.54 for estradiol, Figure 3). Treatment with fulvestrant alone showed no significant difference between cells that were left untreated with fulvestrant (1.2 +/- 1.1, p=0.940). There was no significant difference for cells that were treated with epinephrine alone compared to cells that were treated without epinephrine (0.98 +/- 0.73, p=0.222). Cells that were treated with fulvestrant, estrogen and epinephrine showed no significant difference from any other combination (2.3 +/- 2.4 for equilenin pretreatment and 0.64 +/- 0.47 for estradiol pretreatment, p=0.759). There was no significant difference between cells that were left untreated with estrogens but treated with fulvestrant and epinephrine and those that were not treated with either (1.4 +/- 0.94, p=0.944). Figure 4 shows a collective comparison of all combinations of treatment.

Since treatment with epinephrine did not show any significant effect on cell viability, the data were then analyzed by eliminating epinephrine as a variable to see if there was a significant difference between cells that were treated with fulvestrant and estrogen compared to those without any estrogen treatment. There was no significant difference between cells treated will fulvestrant alone and those treated without fulvestrant (p=0.938). Cells that were treated with estrogens compared to those without any estrogen treatment showed no significance difference (p=0.514).

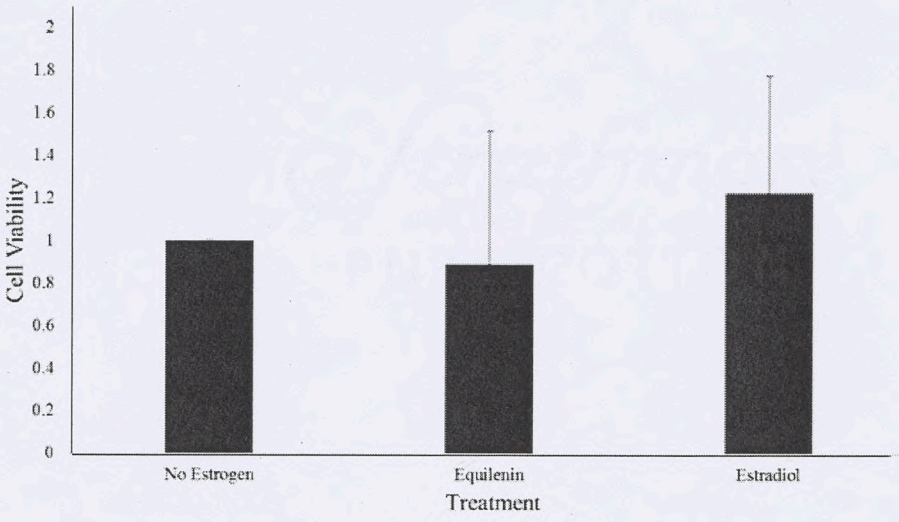

Figure 1. Average astrocyte viability (+/- 1 S. D.) following treatment with 1 μM of estrogen (equilenin or estradiol). Results were not significant.

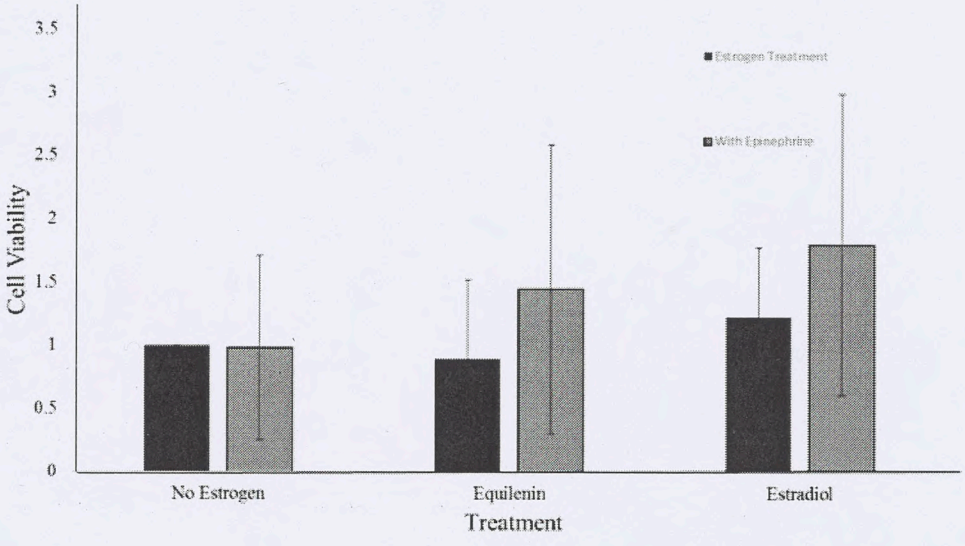

Figure 2. Comparison of average astrocyte viability (+/- 1 S.D.) for cells treated with 1 μM of estrogen (equilenin or estradiol) with cells treated with 1 μM estrogen and 10 μM epinephrine. Results were not significant.

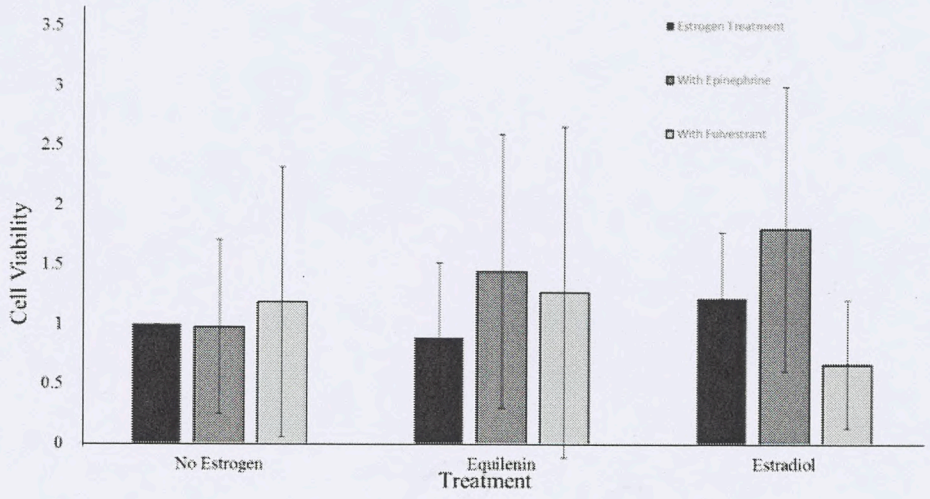

Figure 3. Comparison of average astrocyte viability (+/- 1 S.D.) for cells treated with 1 μM of estrogen (equilenin or estradiol) with cells treated with 1 μM estrogen and 10 μM epinephrine and cells treated with 1 μM estrogen and 1 μM of fulvestrant. Results were not significant.

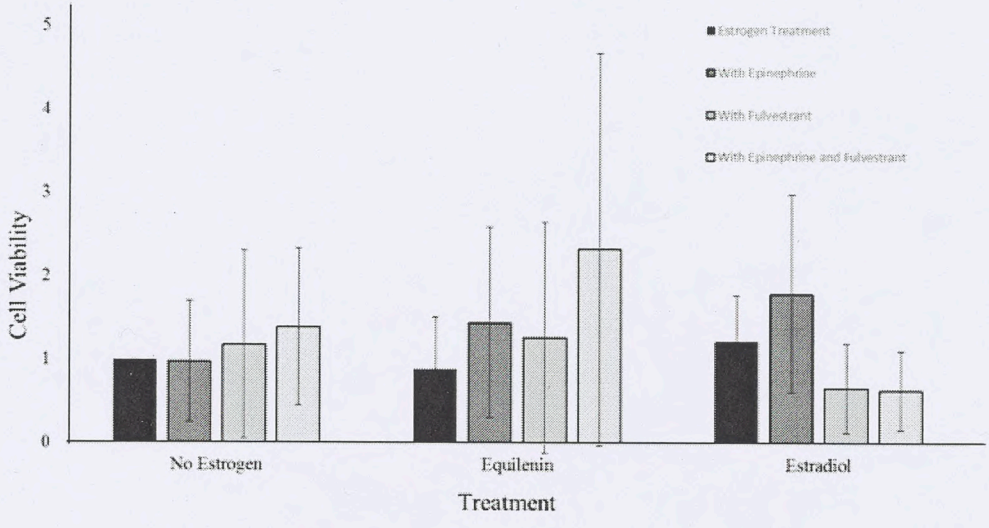

Figure 4. Comparison of average astrocyte viability (+/- 1 S.D.) for cells treated with 1 μM of estrogen (equilenin or estradiol) with cells treated with 1 μM estrogen and 10 μM epinephrine, 1 μM estrogen and 1 μM of fulvestrant and cells treated with 1 μM estrogen, 1 μM of fulvestrant and 10 μM of epinephrine. Results were not significant.

Discussion

Treatment with fulvestrant, epinephrine, and both endogenous estrogens and CEEs showed no significant difference in astrocyte viability compared to the control, which received no treatment. Combinations of any of the treatments also showed no significant difference between each other, even with the eliminating of the epinephrine in analysis. In this particular study, estrogens at a concentration of 1 μM did not provide any benefit or harm to astrocytes at normal conditions for human cultured astrocytes with or without treatment of epinephrine. However, a previous study using a murine *in vitro* model conducted by Grimes and Hughes (2015) showed evidence that estrogens play a role in neuroprotection when oxidatively stressed with hydrogen peroxide (Grimes and Hughes, 2015). While this particular study provided no justification that epinephrine acted as a stressor on astrocytes, studies have been conducted that show that epinephrine and dopamine, another catecholamine, demonstrate toxic effects on neuronal cells at high concentrations (> 1 mM) (Noh et al 1999). The concentration used in this study (10 μM) may have been too low to induce any neurotoxicity.

Fulvestrant prevents estrogen from signaling through transcriptional regulation by completely degrading the ER protein, which is why it is considered to be a complete antagonist of the estrogen receptor (Yeh et al 2013). If estrogens are neuroprotective, then it would be expected that when they are placed under stress, treatment with fulvestrant would show a decrease in cell survival as estrogens are not able to provide any neuroprotection. However, this hypothesis was not supported by the results of this study. This could possibly be due to a different signaling mechanism, in which estrogen could be acting via a second messenger system by utilizing a G-protein coupled receptor in astrocytes. It is also possible that exposure time was not adequate for the fulvestrant or the concentration was too low.

This study was limited in that the number of repetitions and trials were few due to time constraints. There was also only one fixed concentration for each independent variable which limited the range of data that was able to be collected. Data was limited on the effects that fulvestrant has on the central nervous system as most research has focused on breast cancer cell lines as fulvestrant is often used as a therapy for patients who have developed metastatic breast cancer as a result of hormone replacement therapy (Jansen et al 2002). This made it difficult to determine adequate dosing and exposure time for the application of the fulvestrant. It is possible that the fulvestrant did not have enough time to exert its effects on the ER receptor or that the dose was too low to have any significant effects.

Future studies should examine differences in viability for differing concentrations of epinephrine, as well as looking at other catecholamines and their role in astrocyte survival. This study focused solely epinephrine and it would be interesting to examine if there are any differences between epinephrine and other catecholamine derivatives. Epinephrine did not appear to have any significant effect on viability which could be due a concentration that was too diluted or it may not have any effect on neuroglia. Few studies have focused on the neurotoxicity of epinephrine so it is necessary to conduct further treatments to evaluate if it can have a significant effect on viability at different concentrations. Also, exposure time of fulvestrant should be increased as well as experimenting with different concentrations to determine if either has an effect on astrocyte survival. Future studies could also look at measuring oxidative stress as well as effects of oxygen deprivation on viability. Grimes and Hughes (2015) showed that CEEs offer neuroprotection for short term stress with hydrogen peroxide at certain concentrations. While the results of this study did not provide any support for neuroprotection of

either endogenous estrogens or CEEs, varying concentrations and exposure time could provide further insight into the mechanism of estrogen receptor signaling.

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